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Toxicity and Environmental Chemistry of Wastewater from a Kraft Pulp and Paper Mill: Fish Toxicity Studies

Alberta
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TOXICITY AND ENVIRONMENTAL CHEMISTRY OF WASTEWATER FROM A KRAFT PULP AND PAPER MILL: FISH TOXICITY STUDIES

Alberta Environmental Centre

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ABSTRACT

The purpose of this investigation was to determine if effluent discharged from the Procter and Gamble Cellulose Ltd. (Grande Prairie) kraft process pulp and paper mill was deleterious to fish in the Wapiti River. The presence and concentrations of organic and inorganic chemicals in effluent, river water and selected fish tissues were determined. In addition, selected tissues from fish resident in the river adjacent to the mill were examined for evidence of pathological changes.

Measurement of the chemical constituents of upstream and downstream river water and mill effluent in this study was done for the purpose of correlating the chemical constituents of the water with residues in fish. As such, it does not constitute a complete study of the chemistry of either the water or the mill effluent and may not represent the chemical status of the river downstream of the immediate zone of influence of the effluent. However, the results reported herein represent a more in-depth assessment of water and effluent chemistry than is normally carried out for monitoring purposes. For a more complete assessment of downstream water quality, reference should be made to existing water quality and bioassay data.

It has been concluded that:

1. Effluent from the mill was not usually acutely toxic to fish under natural or laboratory conditions.

   Twelve species of fish were found immediately below the waste discharge point compared with ten species in the upstream control area. The age distribution of the species caught above and below the discharge point was similar. Based on the presence of young-of-the-year, at least seven species of fish appeared to reproduce in the vicinity of the mill. Acute toxicity tests of whole mill effluent conducted over a period of several years were usually negative.

2. Effluent from the mill contained alkanes, fatty acids, phenolic compounds, dissolved solids, and high chemical oxygen demand and biological oxygen demand, all commonly associated with pulp mill effluent. Discharge of these constituents lead to a measurable increase in their concentrations in the Wapiti River immediately below the mill.

3. Fish captured between 0.1 - 2.5 km below the mill contained alkanes, fatty acids, chlorophenols and guaiacols, and α,α'-dichlorodimethylsulphone in their tissues at concentrations ranging from trace (detected but not quantifiable) to 5 µg/kg (ppb, wet weight, whole fish), except for

α,α'-dichlorodimethylsulphone which was detected at a concentration of 26 µg/kg. In most cases these compounds were also detected in mill effluent; in a few cases they were also detected in upstream water samples and in fish taken upstream of the mill.

4. Fish captured upstream and downstream of the mill contained concentrations of polychlorinated biphenyls in intestinal fat (but not muscle tissue) at concentrations ranging from trace (detected but not quantifiable) to 5 µg/kg (ppb). Intestinal fat samples of fish captured downstream of the mill also contained similar concentrations of DDT and DDE. These compounds were not detected in mill effluent and the concentrations detected in fish tissues were well below those currently accepted as safe for human consumption.

   Forty other common organic environmental contaminants, primarily pesticides, were analyzed for but not detected in intestinal fat or muscle tissue from fish taken downstream of the mill.

5. Histopathological examination of tissues of small fish resident downstream of the discharge point revealed a slight increase in observable lipid in the livers. This finding suggests a mild unspecified impairment of lipid metabolism and possibly of protein synthesis. The effect of this change on fish production in the river is not known.

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1. INTRODUCTION

1.1 Background Information on the Procter and Gamble Cellulose Ltd. Mill, Grande Prairie, Alberta

A kraft process pulp and paper mill owned by Procter and Gamble Cellulose Ltd. (Grande Prairie) is situated on the north bank of the Wapiti River, approximately 30 km upstream of its juncture with the Smoky River (Figure 1). The mill began operation in 1973 and, in 1980, construction was completed on a 100 million board feet sawmill adjacent to the pulp mill. Wood is harvested mainly from a 13,600 km^2 forest management lease area located south of Grande Prairie.

The pulp mill has a rated capacity of 900 air dried tonnes of pulp per day. Initially, cellulose is removed from lignin by digestion of the chips under heat (170°C) and pressure (~165 psi) in the presence of NaOH and Na_2S. A six stage bleaching process follows using chlorine, sodium hydroxide, hypochlorite and chlorine dioxide as brightening agents. The final stages of production are sheet forming, drying and baling. In total, these processes use 60-94x$10^3 m^3$ of water per day from the Wapiti River. Most of this water is treated and then released back into the river. The effluent treatment facilities consist of the following:

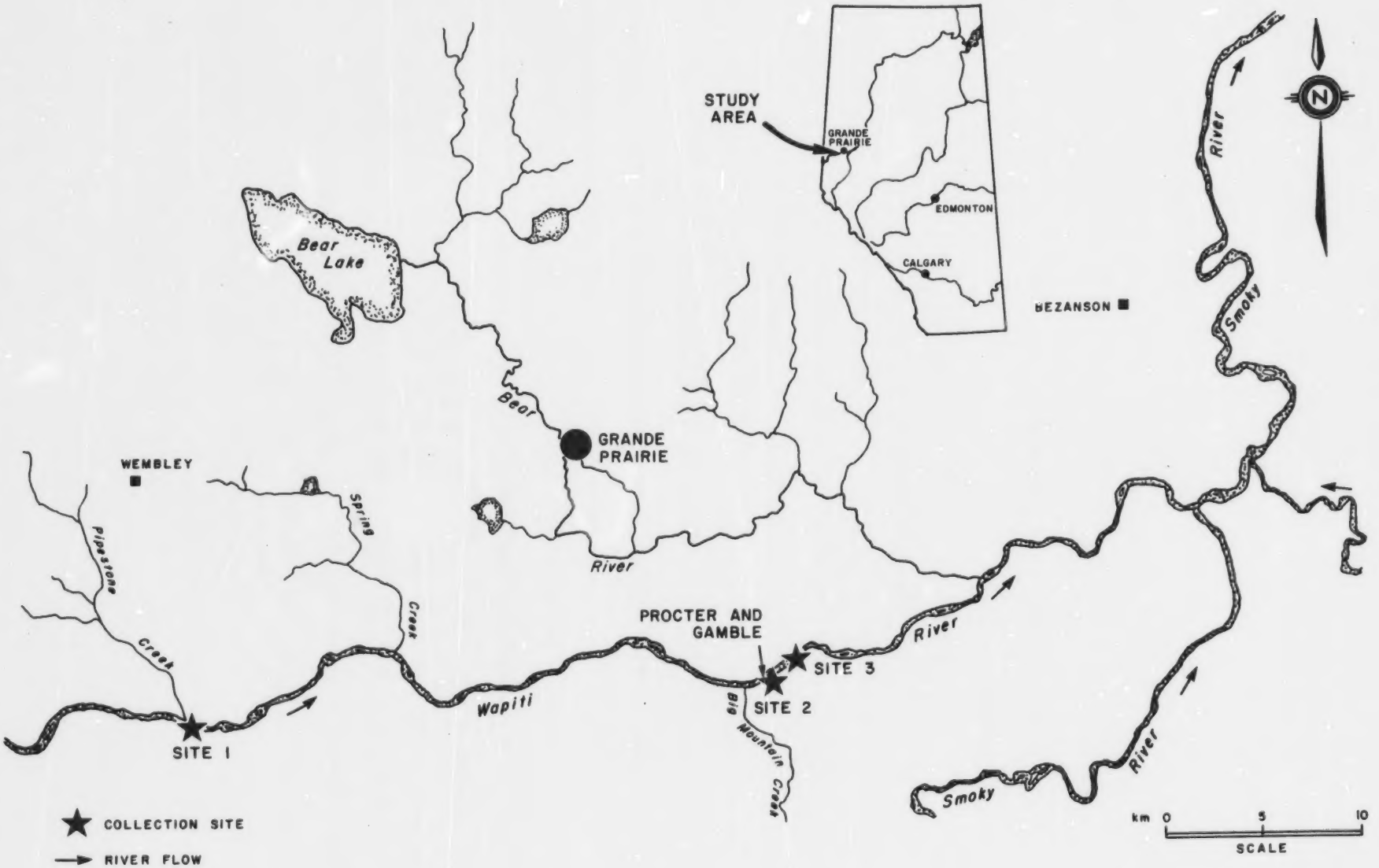

FIGURE 1. STUDY AREA SHOWING COLLECTION SITES.

i) primary clarifier
ii) acid and alkaline neutralization
iii) primary settling basins
iv) aeration lagoon
v) foam retention panel
vi) spill pond

1.2 Purpose of Study

The purpose of this investigation was to determine if effluent from the Procter and Gamble Cellulose Ltd. mill was deleterious to fish collected below the effluent discharge point. Effluent from the holding pond and river water collected from above and below the effluent discharge point were analyzed chemically for organic and inorganic constituents. Fish from above and below the effluent discharge point were analysed for chemical residues and for evidence of pathological changes.

2. METHODS

2.1 Sites and Sample Collection

Three sites were selected for study (Figure 1). Site 1, the control site, was located downstream of the confluence of the Wapiti River and Pipestone Creek. This was approximately 30 km upstream of the Procter and Gamble mill and 63 km upstream of the confluence of the Wapiti and Smoky Rivers. Site 2 consisted of an area from the

effluent diffuser to a point on the river 700 m downstream. Site 3 was located immediately upstream of the Northern Alberta Railway Bridge, about 2.5 km downstream of the effluent diffuser.

The collection of fish, water and effluent was carried out by Alberta Environmental Centre staff during five sampling periods, between June 1, 1981 and October 22, 1982 (Table I).

Table I. Dates of collection of water, fish and effluent.

Date	Site 1		Site 2		Site 3		Effluent
	Water	Fish	Water	Fish	Water	Fish	Pond
June 2-4, 1981	*	*	-	-	*	*	-
July 15, 16, 17, 1981	*	*	*	*	-	-	*
Sept. 29-Oct. 1, 1981	*	*	*	*	-	-	-
May 13-15, 1982	*	*	*	*	-	-	*
October 19-22, 1982	*	*	*	*	-	-	*

- = sample not collected

2.1.1 Water and Effluent Collections

Samples of Wapiti River water were collected on five occasions between June 1981 and October 1982 (Table I). Samples for chemical analysis were preserved by recommended procedures in jars rinsed with

Wapiti River water (Alberta Environment, 1979). All water collections were carried out at Site 1 and 2, except in June of 1981 when Site 2 could not be sampled due to the high water flow. On this occasion only, the water sample was collected at Site 3 located 2.5 km downstream of the mill.

Effluent samples were collected from the final effluent holding pond on July 17, 1981, May 15 and October 21, 1982. Samples were taken in effluent rinsed sample jars and preserved immediately upon collection, as described in Section 2.1.3.

2.1.2 Fish Collections

Fish were taken on each sampling trip at the three sites using an 8 m nylon 0.635 cm mesh minnow seine and similar small mesh dipnets. The live fish were held in aerated river water rinsed polyethylene bags prior to identification. Most of the catch was then frozen at -20°C prior to chemical analysis. A representative number of specimens from each site was preserved in at least one of the following fixative solutions (10% neutral buffered formalin, Rossmans fluid, Bouins and/or ethanol) for histopathological examination. Multimesh gillnets (2.5-10.0 cm mesh) were set approximately halfway between Site 2 and Site 3 to collect fish for pesticide and PCB analysis.

2.1.3 Effluent and Water Sample Preservation:

Organic scan:	preserved by acidifying to pH 1.3 with concentrated H_2SO_4 in a 2 L glass container
Haloforms:	preserved with small amounts of sodium thiosulfate
[1]Routine parameters:	refrigerated in a 750 ml polyethylene bottle
[2]General 1:	refrigerated in a 2 L glass bottle
[3]General 2:	preserved with 1 ml of concentrated H_2SO_4 in a 150 ml polyethylene bottle
Oil and Grease:	preserved with 5 ml of concentrated H_2SO_4 in a 1 L glass bottle
Phenol:	preserved with 5 ml of concentrated H_2SO_4 in a 750 ml glass bottle
Metals:	preserved with 5 ml of concentrated HNO_3 in a 750 ml polyethylene bottle
BOD:	aliquoted from General 1 bottle
Mercury:	preserved with 2 ml of potassium dichromate-nitric acid in a 150 ml polypropylene bottle
Sulfide:	preserved with 1 ml of zinc acetate in a 1 L glass bottle

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[1]Routine parameters includes pH, conductivity, carbonates, total alkalinity, sodium, potassium, chloride, silica, calcium, magnesium, total hardness, sulphate, fluoride, nitrite, nitrate, iron and total dissolved solids.

[2]General 1 includes BOD, odour, total residue, non-filterable residue, filterable residue, turbidity, surfactants, tannins and lignins.

[3]General 2 includes COD, ammonia, phosphorus and total Kjeldahl nitrogen.

2.2 Analysis of Effluent and Water Samples

Samples of effluent and river water were separated by extraction procedures into base/neutral (B/N) and acidic (A) fractions. Separation of the B/N fraction involved extraction of the sample with methylene chloride ($MeCl_2$) twice at pH = 13.0. The organic layer

was then separated and dried with Na_2SO_4 and concentrated to yield the B/N fraction.

The aqueous layer was acidified to pH ≤ 2 and extracted serially with $MeCl_2$. The combined extracts were dried with Na_2SO_4 and then concentrated to yield the acidic (A) fraction.

Analyses for inorganic and other routine water quality parameters were conducted as outlined in the Methods Manual for Chemical Analysis of Water and Wastewaters (Alberta Environment, 1977). Organic analysis was carried out by Chemistry Wing staff using a gas chromatograph/mass spectrometer (GC/MS) instrument.

Due to small sample size, the results of chemical analyses of river water and effluent were not subjected to statistical analysis. However, for each analytical procedure, appropriate quality control procedures including positive and negative controls were used. Results of routine water quality parameters are reported as mg/L (parts per million) except as otherwise specified (Table IV). Results of gas chromatography/mass spectrometry (GC/MS) analysis of effluent and river water for organic residues are reported as µg/L (parts per billion) with a quantifiable detection limit of 0.01 µg/L. Positive findings of <0.01 µg/L are reported as trace concentrations (detected but not quantifiable) (Table V). Details of the extraction and analytical procedures are given in Appendix I.

2.3 Analysis of Fish Tissues for Organic Residues

Flathead chub and longnose suckers captured above and below the effluent diffuser on the September 29-30, 1981, May 13-14, 1982 and October 21, 1982 collection dates were used for organic analysis by gas chromatography/mass spectrometry. Fish were pooled by species and location to arrive at a sample size of approximately nine g. Whole frozen fish were cut into small pieces and thawed at room temperature. The organic fraction was extracted using acetone and hexane, hexane/ether and hydrochloric acid. The organic extract was acidified and concentrated to near dryness using Na_2SO_4. The base/neutral (B/N) fraction was prepared by spiking the concentrated organic extract with d_{10}-phenanthrene and making up to volume.

The aqueous phase was acidified to pH ≤ 2, and extracted with hexane/diethyl ether. The aqueous extract was concentrated to near dryness using Na_2SO_4 as described under B/N fraction. Organic analysis was then carried out by gas chromatography/mass spectrometry.

Because of small sample size, results of chemical analysis of fish tissues for organic residues were not subjected to statistical analysis. However, appropriate quality control procedure were incorporated as part of each methodology. Results of organic analysis of fish tissues by GC/MS are reported as µg/kg (parts per billion) with a quantifiable detection limit of 0.1 µg/kg. Positive findings of less than 0.1 µg/kg are reported as trace concentrations (detected but not quantifiable) (Table V).

Details of the extraction and analytical procedures are given in Appendix II.

2.4 Analysis of Fish Tissues for Pesticides and Polychlorinated Biphenyls

A total of ten longnose suckers (fork length 25.8-49.4 cm) and one northern pike (45.2 cm) collected on the July 16, 1981, September 30, 1981 and October 1, 1981 sampling dates at Site 2 downstream of the effluent diffuser were used for determination of pesticide and polychlorinated biphenyl concentrations. Samples of muscle and fat tissue were removed from each fish, wrapped in aluminum foil and stored at -20°C prior to analysis. Approximately 250 g (wet weight) of combined hypaxial and epaxial muscle tissues and ~5 g of intestinal fat were obtained from each fish.

Twenty-five grams of muscle tissue from each fish was homogenized in the presence of acetonitrile and filtered. The filtrate was extracted with petroleum ether (PE) and the PE layer was separated and evaporated to near-dryness. The PE residue was separated by column chromatography into two fractions which were subsequently analyzed by gas chromatography.

One gram of fat from each fish was homogenized with anhydrous sodium sulfate, fractionated by column chromatography and analyzed by gas chromatography.

Results of organic analysis of fat and muscle tissues by gas chromatography are reported as µg/kg (parts per billion). The detection limit for organochlorine and polychlorinated biphenyl compounds was 5 µg/kg; the detection limit for organophosphate, phenoxy, sulphone and nitrile compounds was 50 µg/kg (Table VI).

Details of extraction procedures for muscle and fat, the perchlorination step for improving the detection limit for polychlorinated biphenyls and the analysis with precision and accuracy data are given in Appendix III.

2.5 Histopathological Evaluation of Fish Tissues

A total of 43 lake chub (21 control from Site 1, 22 principal from Sites 2 and 3) and 33 longnose suckers (16 control, 17 principal) collected in July 1981, September 1981 and May 1982 were submitted for histopathological evaluation (Table I). Fish of both species ranged in length and weight from 3.1 to 10.8 cm and 0.2 to 12.8 g respectively (Table II). All specimens were fixed in formalin, except those collected in June 1981 which were fixed in Bouins. The livers of all specimens except those collected in June and July 1981 were dissected out and fixed for special stains. Livers from the September 1981 collection were split and fixed in both ethanol and formalin, while livers from the May and October 1982 collection were split between Rossmans fluid (Humason, 1979) and formalin. Tissues (brain, gills, liver, kidney, spleen, gonad, skeletal muscle, meninges) from

all fish were processed routinely, stained with hematoxylin and eosin (H&E) and examined by light microscopy.

The following special fixatives and stains for lipid and glycogen were used on sections of liver from selected fish to augment and verify observations made with routine formalin fixed, haematoxylin and eosin (H&E) stained sections. Best's carmine and Periodic acid-Schiff

Table II. Length/weight data of fish used in histopathological examination.

TRIP DATE	STATUS OF SAMPLE	SPECIES	SAMPLE SIZE	FORK LENGTH (cm)	WET WEIGHT (g)
July 1981	control	Lake chub	8	5.0(4.0-5.8)[a]	1.2(0.8-1.5)[b]
		Longnose sucker	5	7.1(6.1-7.8)	4.4(2.6-5.3)
	principal	Lake chub	10	6.8(4.7-9.6)	2.5(1.5-2.9)[c]
		Longnose sucker	6	5.7(5.3-5.9)	2.8(1.9-4.5)
Sept. 1981	control	Lake chub	5[d]	3.2(3.1-3.4)	0.3(0.2-0.4)
		Longnose sucker	5[e]	4.5(4.3-4.7)	0.9(0.7-1.1)
	principal	Lake chub	5	8.5(8.0-9.3)	6.2(4.8-8.3)
		Longnose sucker	5	8.6(5.6-10.7)	7.7(2.8-12.8)
May 1982	control	Lake chub	8	5.6(4.5-7.0)	1.6(0.6-3.0)
		Longnose sucker	6	5.9(5.3-7.5)	1.7(1.0-3.4)
	principal	Lake chub	7	9.9(9.1-10.8)	9.8(7.3-12.8)
		Longnose sucker	6	8.6(7.2-11.0)	5.7(3.1-10.9)

[a]mean length and range
[b]based on two measurements
[c]based on five measurements
[d]five fish not suitable for histopathological evaluation
[e]two fish not suitable for histopathological evaluation

(PAS) (with and without diastase digestion) stains were used on formalin-fixed livers from 10 lake chub (five principal, five control) and eight longnose suckers (five principal, three control). Best's carmine, PAS (without diastase digestion) and haematoxylin and eosin stains were used on all livers fixed in Rossmans fluid. Best's carmine stain was also used on livers fixed in ethanol. Oil Red O stain was used on frozen liver sections.

The amounts of glycogen and lipid in hepatocytes were determined in a semi-quantitative manner on a scale of 0 to 5+ (0 = absent, 1+ = slight or questionable, 2+ = clearly present but not conspicuously so, 3+ = marked, 4+ = severe, 5+ = extreme).

Microscopic analysis was done without prior knowledge of the treatment to avoid bias. Differentiation between glycogen and lipids was based on the morphological appearance of the intracytoplasmic vacuoles in carmine stained sections of ethanol fixed liver compared to formalin fixed liver.

2.6 Effects of Food Supply on Liver Lipid Concentrations

In order to determine the effect of reduced food supply on the amount of lipid in fish livers, collections of river water, gravel, lake chub and longnose suckers were made from Site 1 on October 21 and 22, 1982. The seined fish were placed in rinsed polyethylene bags partially filled with Wapiti River water; medical grade oxygen was

introduced and the bags were tied shut. The bags were then placed in coolers with ice for transport to the laboratory.

Wapiti River water (568 L) at 1°C was collected in five clean polyethylene lined barrels as well as gravel and rock substrate. Upon return to the laboratory, washed gravel and rock substrate were placed in a living stream unit as well as three aquariums, which were then filled with river water. The fish were gradually added to the tanks and two of the aquariums were acclimated to a mean temperature of 9.5°C, with the remainder of the fish maintained in a controlled environment room at at mean temperature of 3.5°C to simulate seasonal river temperatures (Table III).

Table III. Water quality for fish held at 3.5°C.

Parameter	Mean	N	Range
Dissolved oxygen (mg/l)	11.1	70	8.4 - 13.9
Temperature (°C)	3.5	84	3.0 - 4.0
pH	8.2	70	7.3 - 8.7
Conductivity, µmhos	580.1	70	455 - 697

Water quality for fish held at 9.5°C

Parameter	Mean	N	Range
Dissolved oxygen (mg/l)	9.49	61	6.3 - 11.3
Temperature (°C)	9.61	72	7.6 - 10.8
pH	8.02	60	6.8 - 8.5
Conductivity, µmhos	396.2	61	328 - 437

N = number of measurements

Food was withheld from the fish for 12 consecutive weeks. Each week, three lake chub were taken from the living stream, killed by severing the spinal cord and preserved in formalin. The tissues were then stained with H&E and examined by light microscopy.

3.0 RESULTS

3.1 Fish Species and Age Distribution

A total of twelve species of fish were found immediately below the mill. These included burbot Lota lota, northern pike Esox lucius, lake chub Couesius plumbeus, flathead chub Hybopsis gracilis, pearl dace Semotilus margarita, slimy sculpin Cottus cognatus, longnose sucker Catostomus catostomus, an unidentified salmonid, longnose dace Rhinichthys cataractae, mountain whitefish Prosopium williamsoni, redside shiner Richardsonius baltatus, and trout perch Percopsis omiscomaycus. All of the above species were found above the mill with the exception of pearl dace and the unidentified salmonid.

Young-of-the-year and 1+ years of lake chub, flathead chub, pearl dace, and longnose sucker were collected, indicating that these species may spawn in the vicinity of the mill. The pike and burbot were >2 years. Although direct estimates were not made of population numbers, the age distribution of species caught at Site 1 was comparable to that at Site 2. Fine netting was used at both sites and number of fish caught per unit time was similar.

3.2 Chemical Analysis of Wapiti River Water

3.2.1 Analysis of Wapiti River Water for Routine Water Quality Parameters

River water samples were analyzed for a total of 47 standard water quality parameters (Table IV). Of these, 15 were non-specific but commonly measured water quality parameters, 20 were metals or metalloids, eight were anionic and four were organic compounds. Results of analyses were compared with recommended Canadian Drinking Water Quality and Aquatic Life Standards (Environment Canada, Water Quality Source Book, 1979) and other documents (US EPA, 1984-85) where possible.

A total of 43 water quality parameters were detected in water taken from Site 1 (upstream of the mill). Dissolved organic carbon, aluminum, chloroform and dichlorobromoethane were assayed for but not detected. The routine water quality profile for water upstream of the mill was otherwise unremarkable.

A total of 45 water quality parameters were detected in water taken from Sites 2 and 3 (downstream of the mill). Chloroform and dichlorobromoethane were assayed for but not detected.

The concentration of 30 water quality parameters were elevated in downstream water; 17 parameters were not elevated. Of the 30 compounds which were elevated in downstream water, 6 were only slightly elevated (a twofold increase or less); 9 were moderately elevated (a two to fivefold increase); 7 were markedly elevated (a

five to tenfold increase); and 8 compounds were very markedly elevated (more than a tenfold increase). Parameters which were markedly or very markedly elevated (a fivefold increase or greater) included conductivity, total dissolved solids, filterable residue, dissolved organic carbon, biological oxygen demand, chemical oxygen demand, total nitrogen, surfactants, aluminum, total mercury, potassium, sodium, chloride, sulfate and phenols.

3.2.2 Analysis of Wapiti River Water for Organic Residues

River water samples were scanned for residues of organic compounds including acidic compounds, phenolic compounds and neutral compounds by gas chromatography/mass spectrometry (Table V).

Six organic compounds were detected in water samples taken upstream of the mill. Of these, one, palmitic acid, was also detected in effluent, in downstream water and in fish taken both upstream and downstream of the mill. Palmitic acid is a fatty acid generally resulting from natural decay of biological materials.

The remaining five compounds detected in upstream water samples were not detected in effluent, downstream water or upstream or downstream fish tissues. These included three low molecular weight fatty acids, benzothiozole and a low molecular weight aliphatic hydrocarbon. These compounds were considered to be most likely of biological origin.

A total of 19 organic compounds were detected in water samples taken downstream of the mill. Six of the 19 were also detected in

mill effluent. Two of these six compounds were naturally occurring fatty acids and two were phenol compounds, all detected at trace (detected but not quantifiable) concentrations. Guaiacol was detected at a concentration of 0.1 - 0.5 µg/L and methoxycyclohexadiene at 0.01 - 0.1 µg/L.

Thirteen of the compounds detected in downstream water samples were not detected either in mill effluent or in upstream water. These compounds (except for trichlorobiphenyl and tetrachlorobiphenyl) were considered to be most likely derivatives of compounds which had been detected in mill effluent. The majority of these compounds were organochlorines; however, toluene and xylene were also detected. Methylene chloride was detected at a concentration of 12 µg/L and chloroform at a range of 2-6 µg/L; the remaining 11 compounds were detected in concentrations of 1 µg/L (part per billion) or less.

The origins and environmental fate of these compounds are discussed in 4.3.

3.3 Chemical Analysis of Mill Effluent

3.3.1 Analysis of Mill Effluent for Routine Water Quality Parameters

A total of 46 water quality parameters were detected in mill effluent (Table IV).

Most nonspecific parameters (excluding pH) were elevated in comparison to upstream water samples primarily due to increased

Table IV. Water quality profile of mill effluent and of Wapiti River Water above and below effluent discharge point. Concentrations expressed as mg/L[1] (except as specified).

Parameter	Standards[2,3] Drinking Water	Standards[2,3] Aquatic Life	Upstream (Site 1) Control Mean	Upstream (Site 1) Control Range	Effluent Mean	Effluent Range	Downstream (Site 2 and 3) Mean	Downstream (Site 2 and 3) Range
Non-Specific:								
1. pH	6.5 - 8.0	6.5 - 9.0		(7.9 - 8.6)		(7.2 - 7.6)		(7.7 - 8.1)
2. Conductivity, µmohs	-	-	265.0	(198 - 398)	5025.0	(4980-5070)	2152.0	(196 -5550)
3. Total Hardness	500	-	122.5	(81 - 183)	566.0	(560- 577)	306.0	(78 - 662)
4. Total Alkalinity	-	720	119.6	(90 - 157)	196.0	(90- 357)	457.0	(414 - 500)
5. Total Dissolved Solids	500	-	145.0	(105 - 211)	2619.0	(2465-2779)	1124.0	(94 -2867)
6. Total Residue (Calc.)	-	-	559.0	(275 - 905)	4571.0	(4178-4983)	1705.0	(478 -4350)
7. Filterable Residue	-	-	262.0	(130 - 390)	4245.0	(3880-4610)	1520.0	(160 -4330)
8. Non-filterable Residue	-	-	297.0	(3 - 515)	326.0	(298- 353)	181.0	(20 - 380)
9. Dissolved Inorganic Carbon	-	-	31.6	(26 - 39)	100.0	(71- 130)	45.0	(26 - 77)
10. Dissolved Organic Carbon	-	-	ND	ND	258.0	(2100- 305)	125.0	(12 - 350)
11. Biological Oxygen Demand	-	-	1.6	(<1.0- 3.0)	80.0	(74- 85)	35.8	(1.6- 133)
12. Chemical Oxygen Demand	-	-	29.9	(<5.0-58.3)	982.0	(964-1000)	432.6	(51.6-1062.5)
13. Total Nitrogen	-	-	0.792	(.130-1.57)	7.213	(5.8-8.625)	3.355	(0.400-7.900)
14. Tannins and Lignins	-	-	1.8	(1.8, 1.8)	91.00	(90, 92)	1.55	(1.4-1.7)
15. Surfactants	-	-	0.08	(0.05-0.14)	11.14	(8.53-13.75)	4.49	(0.13-14.42)
Metals and Metalloids:								
1. Aluminum	-	-	ND[4]	ND	1.348	(1.21-1.50)	4.506	(1.160-9.110)
2. Arsenic	0.05	-	0.0024	(<0.002-0.0058)	0.0011	(0.0004-0.0019)	0.0027	(0.004-0.0056)
3. Beryllium	-	0.01	<0.001	0	ND	ND	<0.001	0
4. Cadmium	0.005	0.001	0.001	(<0.001-0.002)	0.004	(0.002-0.007)	<0.003	(<0.001-0.006)
5. Calcium	-	-	37.3	(26-57)	202	(198-206)	107.0	(25-239)
6. Chromium	0.05	0.1	0.009	(<0.001-0.020)	0.013	(<0.001-0.025)	0.020	(0.011-0.029)
7. Cobalt	-	-	0.004	(<0.001-0.007)	0.003	(0.003-0.003)	<0.004	(<0.001-0.007)
8. Copper	1.0	[a]	0.014	(<0.001-0.032)	0.013	(0.009-0.018)	0.010	(0.005-0.013)
9. Iron	0.3	1.0	0.99	(0.044-1.86)	0.865	(0.83-0.93)	1.53	(0.99-1.77)
10. Lead	0.05	6	0.036	(<0.003-0.195)	0.109	(0.003-0.256)	<0.057	(<0.003-0.272)
11. Molybdenum	-	-	0.005	(<0.001-0.009)	0.003	(<0.001-0.004)	<0.006	(<0.001-0.009)
12. Magnesium	-	-	6.0	(4-10)	15.0	(14.0-16.0)	10.0	(4-16)
13. Manganese	0.05	-	0.096	(<0.08-0.195)	0.644	(0.627-0.650)	0.375	(0.051-0.932)
14. Mercury	0.001	0.00005	<0.0001	(<0.0001)	<0.0001	(<0.0001)	0.0059	(<0.0001-0.0200)
15. Nickel	-	1% LC50[5]	0.124	(<0.001-0.020)	0.015	0.014-0.017	0.024	(0.007-0.071)
16. Potassium	-	-	0.8	(0.5-1.2)	11.875	(9.0-15.0)	5.4	(0.9-13.3)
17. Selenium	0.01	1% LC50	0.005	(<0.002-0.0005)	(<0.0002)	(<0.002)	<0.002	(<0.0002-0.003)
18. Sodium	-		7.0	(3-13)	785.0	(685.0-840.0)	308.0	(4-833)
19. Vanadium	-	1% LC50	0.013	(<0.002-0.027)	0.008	(0.008-0.008)	0.018	(0.007-0.027)
20. Zinc	5.0	-	0.044	(0.004-0.083)	0.177	(0.111-0.212)	0.088	(0.017-0.155)

[1] mg/L equals parts per million
[2] Environment Canada, Water Quality Source Book, 1979
[3] US EPA, Criteria Documents, 1984-85
[4] Detection limit of 0.02 mg/L
[5] 1% of 96 h LC_{50} of sensitive indigenous species
[6] 10% of 96 h LC_{50} of sensitive indigenous species
[7] Total halomethanes
ND - not detected; blank means no data; dash means no standard
[a] Essentially free and non-tainting

Table IV. Water quality profile of mill effluent and of Wapiti River Water above and below effluent discharge point. Concentrations expressed as mg/L[1] (except as specified).

Parameter	Standards[2,3] Drinking Water	Standards[2,3] Aquatic Life	Upstream (Site 1) Control Mean	Upstream (Site 1) Control Range	Effluent Mean	Effluent Range	Downstream (Site 2 and 3) Mean	Downstream (Site 2 and 3) Range
Anions:								
1. Bicarbonate	-	-	141.8	(110-179)	491.0	(484-504)	238.0	(110-436)
2. Chloride	-	-	1.6	(<1-3)	943.0	(903-963)	422.0	(<1-1161)
3. Fluoride	1.5	-	0.09	(0.07-0.12)	0.10	(0.08-0.14)	0.09	(0.07-0.12)
4. Nitrate/Nitrite	10	-	0.05	(<0.05-0.07)	0.08	(<0.05-0.25)	0.10	(<0.05-0.20)
5. Nitrite	1.0	-	0.05	(<0.05-0.06)	0.18	(<0.05-0.14)	0.12	(<0.005-0.25)
6. Silica	-	-	2.9	(2.7-3.2)	10.0	(9.0-10.6)	5.5	(3.0-10.0)
7. Sulphide	-	-	0.32	(<0.02-0.76)	1.46	(1.25-1.64)	0.90	(0.72-1.11)
8. Sulphate	500	-	19.3	(10-34)	468.0	(405-500)	155.6	(<5.0-390)
Organic Compounds:								
1. Phenols	0.002	0.001	0.004	(<0.002-0.008)	1.00	(0.212-1.40)	0.09	(0.008-0.240)
2. Oil and Grease		10% LC50[6]	1.3	(<0.2-2.3)	1.20	(0.5-2.5)	1.1	(<0.2-2.2)
3. Chloroform ($CHCl_3$)	0.35[7]	-	ND	ND	0.071	(0.063-0.080)	ND	ND
4. Dichlorobromomethane ($CHCl_2Br$)	-	-	ND	ND	0.0015	(<0.001-0.002)	ND	ND

[1] mg/L equals parts per million
[2] Environment Canada, Water Quality Source Book, 1979
[3] US EPA, Criteria Documents, 1984-85
[4] Detection limit of 0.02 mg/L
[5] 1% of 96 h LC_{50} of sensitive indigenous species
[6] 10% of 96 h LC_{50} of sensitive indigenous species
[7] Total halomethanes
ND - not detected; blank means no data; dash means no standard
[8] Essentially free and non-tainting

concentrations of suspended and dissolved solids. Surfactants, chemical oxygen demand and dissolved organic carbon were most elevated; tannins and lignins, biological oxygen demand, filterable residue, total dissolved solids and conductivity were also elevated at least tenfold.

The concentrations of metals and metalloids were generally low. Thirteen of twenty metals or metalloids were not elevated in comparison to upstream water samples; 5 were slightly elevated. Only calcium and sodium were markedly elevated in the effluent, probably due to process chemicals used in the mill. The concentrations of aluminum and mercury were lower in the effluent than in downstream water. The higher downstream concentrations of these metals may have resulted from physical disturbance of natural concentrations of these metals in the river sediment rather than from the chemical composition of mill effluent. Bicarbonates, chlorides, sulphates and phenols were also elevated in comparison to upstream water samples.

3.3.2 Analysis of Mill Effluent for Organic Residues

Sixty-one organic compounds were detected in mill effluent at concentrations ranging from trace (detected but not quantifiable) to a maximum of 400 µg/L by GC/MS analysis (Table V). Of the 61 compounds identified, 31 were present in concentrations of 5 µg/L or less, 16 at concentrations between 5 and 25 µg/L, seven at concentrations between 25 and 100 µg/L and seven in concentrations

Table V. Organic Compounds Tentatively Identified in Effluent, Water and Fish by GC/MS.

Compound	In Effluent µg/L[b]	River Water µg/L		Fish µg/kg[c]	
		Upstream	Downstream	Upstream	Downstream
Acidic Compounds:					
1. Chlorobenzoic acid	1-5				
2. $C_8H_8O_2$ aromatic acid (MW 136)	5-150				
3. Myristic acid	1-5				
4. Palmitic acid	1-5	0.1-0.5	[a]	[a]	[a]
5. Stearic acid	1-5		[a]	[a]	[a]
6. Dicarboxylic acid (MW 188)	1-5				
7. $C_{15}H_{30}O_2$ fatty acid (MW 242)		0.1-0.5			
8. $C_{16}H_{32}O_2$ fatty acid (MW 256)		0.1-0.1			
9. 3-Chlorohexanoic acid			[a]		
Phenolic Compounds:					
1. Substituted phenol (MW 170)	1-5				
2. Methyl ethyl phenol	1-5				
3. Methyl t-butyl phenol	10-15				
4. Methyl isopropyl phenol	10-15		[a]		
5. Trichlorophenol	1-15		[a]		4
6. Tetrachlorophenol	1-15				2
7. Substituted catechol (MW 168)	10-15				
8. Acetocatechol	1-5				
9. Guaiacol	10-75		0.1-0.5		
10. Methyl guaiacol	1-5				
11. Trichloroguaiacol	1-15				1.4
12. Acetoguaiacol	1-5				
13. Acetosyringol	1-5				
14. Acetothiophene	1-5				
15. Ethyl acetothiophene	1-5				
16. Methyl ethyl thiophene	1-5				
Neutral Compounds:					
1. Veratrole	1-5				
2. Dimethyl benzyl benzene	1-5				
3. Dimethyl benzyl alcohol	1-5				
4. Ethyl dimethyl benzene	1-5				
5. Acetophenone	50-75				
6. Dimethyl dihydrobenzene	1-10				
7. Terpineol	5-10				
8. Diethyl thiophene	1-5				
9. C_9H_4S isomer (MW 154)	1-5				
10. Dimethyl sulphone	1-10				
11. Dichlorodimethyl sulphone	10-75				5.2
12. Dimethyl disulphone	75-250				
13. Dimethyl trisulphone	25-300				
14. Dimethyl sulphide	25-100				

[a]not quantified; blank means not found.
[b]detection limit in effluent and river water approximately 0.01 µg/L (ppb)
[c]detection limit in fish tissues approximately 0.1 µg/kg (ppb)

Table V. Organic Compounds Tentatively Identified in Effluent, Water and Fish by GC/MS.

Compound	In Effluent µg/L[b]	River Water		Fish	
		Upstream µg/L	Downstream	Upstream µg/kg[c]	Downstream
15. $C_9H_{12}O$ isomers (MW 136)	25-250				
16. Monochloro organic (MW 122)	1-10				
17. Dichloropropyl substituted organic (MW 186)	5-10				5.2
18. Dimethyl cyclopentadiene	1-10				
19. 2 methyl 2 cyclo pentene 1-One	1-5				
20. 3 methyl 2 cyclo pentene 1-One	1-5				
21. Dimethyl hexadiene	1-25				
22. Ethyl dimethoxy hexadiene	1-5				
23. Cyclohexane	1-10				
24. Bicyclohexane	1-5				
25. Dimethyl ethyl cyclohexadiene	1-5				
26. Tetramethyl cyclohexadiene	100-150				
27. Isopropyl methyl cyclohexadiene	350-400				
28. Chloromethyl cyclohexadiene	1-5				
29. Methoxy cyclohexadiene	1-25		0.01-0.1		
30. Cyclohexenone	1-10				
31. Methyl isopropyl cyclohexanol	1-5				
32. Isopropyl methyl cyclohexanol	1-5				
33. Trimethyl cyclohexanol	10-50				
34. Trimethyl bicyclo (2,2,1) heptanone	1-50				
35. Camphor	1-5				
36. $C_{10}N_{16}$ isomers (MW 136)	50-250				
37. $C_{10}H_{18}$ isomers (MW 138)	75-100				
38. $C_{16}H_{34}$ alkane branched	[a]				
39. $C_{17}H_{36}$ alkane branched	[a]				
40. $C_{11}H_{14}O_2S_2$ fatty acid (MW 258)		1-1.5			
41. Benzothiozole		0.01-0.1			
42. Aliphatic hydrocarbons (MW 150-350)		0.1-0.5			
43. Monochloro aromatic acid (MW >210)			0.01-0.1		
44. Substituted methyl ester			0.5-1		
45. Molecular sulphur S_n			0.01-0.1		
46. Chloroform			2-6		
47. Methylene chloride			12		
48. $C_{10}H_{14}$ (MW 136)			0.01-1.0		
49. Toluene			0.1		
50. Xylene			0.1-1.0		
51. 1,1 dichloroethylene			0.01		
52. Carbon tetrachloride			0.01-0.1		
53. Chlorobenzene			0.01-0.1		
54. Trichlorobiphenyl			0.01-0.1	[a]	
55. Tetrachlorobiphenyl				[a]	

[a] not quantified; blank means not found.
[b] detection limit in effluent and river water approximately 0.01 µg/L (ppb)
[c] detection limit in fish tissues approximately 0.1 µg/kg (ppb)

between 100 and 400 µg/L. Six of these compounds were acidic in nature, 16 were phenolics and 39 were neutral compounds.

The origins and environmental fate of these organic residues are discussed in 4.3.

3.4 Chemical Analysis of Fish Tissues

3.4.1 Analysis of Fish Tissues for Organic Residues

Tissue homogenates of whole fish resident in the Wapiti River above and below the mill were analyzed by gas chromatography/mass spectrometry for residues of acidic, phenolic and neutral compounds (Table V). Secondary analysis of selected compounds was carried out using Selected Ion Monitoring (Table VII).

Four organic compounds were identified in tissues of fish taken upstream of the mill by GC/MS. Two of these were fatty acids detected at trace (detected but not quantifiable) concentrations and presumably of natural origin. The remaining two compounds were trichlorobiphenyl and tetrachlorobiphenyl, both of which were also detected at trace concentrations. One additional compound (α,α'-dichlorodimethyl-sulphone), was detected by SIM at a concentration of 10 µg/kg.

Seven organic residues were detected in tissues of fish taken downstream of the mill. Of these, two were fatty acids detected in trace quantities and presumably of natural origin. The remaining compounds were trichlorophenol, tetrachlorophenol, trichloroguaiacol

and an unidentified monochloro organic compound of low molecular weight, all of which were detected at concentrations of approximately 5 µg/kg or less. The presence and concentrations of these compounds were confirmed by SIM. α,α'-dichlorodimethylsulphone was detected at a concentration of 5.2 µg/kg by GC/MS and a concentration of 26 µg/kg by SIM.

Of the nine compounds detected in fish tissues, five have a half-life greater than ten days and can be considered persistent. These are trichlorophenol, tetrachlorophenol, trichloroguaiacol, trichlorobiphenyl and tetrachlorobiphenyl.

The three persistent organic residues found in downstream fish (trichlorophenol, tetrachlorophenol, trichloroguaiacol) were also detected in mill effluent. The two persistent residues found in upstream fish by GC/MS (trichlorobiphenyl and tetrachlorobiphenyl), were not detected in mill effluent or downstream fish. However, the presence of specific polychlorinated biphenyls was confirmed by gas chromatography in tissues of fish taken downstream of the mill (Table VI).

Table VI. Concentration of targeted compounds in fish from Sites 2 and 3 combined.

Compound Detected	Concentration µg/kg
1. Monochlorosubstituted organic compound	5.2
2. Trichlorophenol	4.0
3. Tetrachlorophenol	2.0
4. Methoxy ethoxy trichloroguaiacol	1.4
5. α,α'-Dichlorodimethylsulphone*	26.0

*reported concentration net of background; concentration of ~10 µg/kg in upstream (control) fish

3.4.2 Analysis of Fish Tissues for Pesticides and Polychlorinated Biphenyls

Muscle and intestinal fat tissue samples from 11 individual fish captured at Site 2 below the effluent diffuser were screened for pesticides and polychlorinated biphenyls by gas chromatography (Table VI). The following compounds were detected at concentrations close to the detection limit of 5 µg/kg (ppb) in fat tissues of fish taken both upstream and downstream of the mill. They were not detected in samples of muscle tissue.

DDT (4,4'-dichlorodiphenyltrichloroethane)

DDE (4,4'-dichlorodiphenyldichloroethylene)

Aroclor 1254

Aroclor 1260

Aroclor 1242

A total of 22 organochlorine compounds were assayed for at a detection limit of 5 µg/kg and were not detected in either muscle or

Table VII. Pesticides and PCB's analyzed for but not detected in intestinal fat and muscle tissue of fish in this study.[1]

Chlorinated Compounds[2]	Organophosphate Pesticides[3]	Phenoxy Pesticides[3]	Other[3]
HCB (hexachlorobenzene)	Parathion	MCPA (metachlorophenoxy acetic acid)	Tedion[4]
γ,β, α HCH (hexachlorocyclohexane, or BHC)	Diazinon	2, 4-D	Deconil[5]
Heptachlor	Sumithion	2, 4, 5-T	
Heptachlor epoxide	Malathion	Picloram	
Chlordane (cis)	Ethion	MCPB (metachlorophenoxy butyric acid)	
Oxychlordane	Dioxythion	Silvex	
Aldrin	EPN (O-ethyl, O-4-nitro-phenyl phenyl phosphonotiolate)	Dicamba	
Dieldrin	Dursban	Pentachlorophenol	
p,p-DDD			
Dicosol			
Polybrominated Biphenyls			
Mirex			
Pentachlorobenzene			
Toxaphene			
Methoxychlor			
Perthane			
Telodrine			
PCNB (Pentachloronitrobenzene)			
TCNB (Tetrachloronitrobenzene)			
Endosulfan			
Thiodan I			
Endrin			

[1]DDT, DDE and 3 polychlorinated biphenyls were detected in fat but not muscle tissue at concentrations close to the detection limit of 5 mg/kg (ppb) (Section 3.2.4)
[2]Detection limit approximately 5 µg/kg (ppb)
[3]Detection limit approximately 50 µg/kg (ppb)
[4]Sulphone group acaricide
[5]Nitrile group fungicide

fat tissue. In addition, eight organophosphorus compounds, eight phenoxy compounds, a sulphone acaricide and a nitrile fungicide were assayed for but not detected at a detection limit of 50 µg/kg (ppb).

3.5 Histopathological Evaluation of Fish Tissues

3.5.1 General Observations

Forty-three lake chub were examined including 21 taken from Site 1 upstream of the mill (controls) and 22 from Sites 2 and 3 downstream of the mill (principals). Five of the control livers were unsuitable for examination due to inadequate sample size or autolytic changes.

Thirty-three longnose suckers were examined including 16 control fish and 17 principals. Two of the control fish were also unsuitable for examination.

Generally brain and meninges, liver, kidney, spleen, gonad and skeletal muscle were examined.

Differences between control and principal fish were minimal in most cases and limited to relative differences in observable hepatic glycogen and lipid. In a few fish, these differences were pronounced.

3.5.2 Hepatic Glycogen Content

In lake chub livers, glycogen content of downstream (principal) fish was about half that of upstream controls. Seven of 16 control fish had a glycogen content of two or less measured on a five-point

scale (Table VIII), while nine had a glycogen content greater than two. By contrast, 19 of 22 principal fish had a glycogen content of two or less while only three had a glycogen content greater than two. Glycogen content was slightly higher in both principal and control fish during the mid-summer feeding season. The greatest difference in glycogen content was in fish taken early in the year (May) when principals had only about one-quarter the glycogen content of controls.

In longnose suckers, glycogen content of downstream principals was about two-thirds that found in controls. Of 14 control fish, five had a glycogen content of two or less while nine were greater than two. Of 16 principal fish, nine had a glycogen content of two or less while eight were greater than two. The lowest glycogen content was observed in both control and principal fish taken during July.

3.5.3 Hepatic Lipid Content

No visible lipid was found in control (upstream) fish of either species (Table VIII). Tissues from five control lake chub taken in October were not suitable for examination.

Visible hepatic lipid was also not found in 12 of 22 principal (downstream) lake chub. However, seven of these 12 were taken in May at the end of the dormant period. Ten of 15 fish taken during the feeding season did show evidence of visible lipid.

By contrast, only three of 17 principal longnose suckers showed evidence of visible lipid and the degree of lipidosis was much less pronounced than in the lake chub.

Table VIII. Relative amounts of glycogen and lipid in hepatocytes of lake chub and longnose sucker collected from the Wapiti River, Alberta, 1981-82.

	Glycogen						Lipid					
	Relative Amount						Relative Amount					
Fish Species	0	1+	2+	3+	4+	5+	0+	1+	2+	3+	4+	5+
Lake chub (control	1	3	3	7	2	-	16	-	-	-	-	-
Lake chub (principal)	9	2	8	2	1	-	12	1	6	1	2	-
Longnose sucker (control)	1	-	4	3	3	3	14	-	-	-	-	-
Longnose sucker (principal)	5	-	4	5	3	-	14	1	2	-	-	-

*0 = Absent
1+= Slight or questionable
2+= Clearly present but not conspicuous
3+= Marked
4+= Severe
5+= Extreme
- = No data

3.5.4 Histochemistry

The findings of the special staining procedures did not add to the information provided by the H&E sections, but were useful in verifying the distinction between lipid and glycogen in questionable cases. The numerical values for glycogen were higher for both control and principal fish in formalin fixed PAS stained sections. However, the relative difference between controls and principals was consistent. The best distinction between glycogen and lipid was provided by ethanol fixed carmine stained livers; the numerical results correlated well with formalin fixed H&E sections.

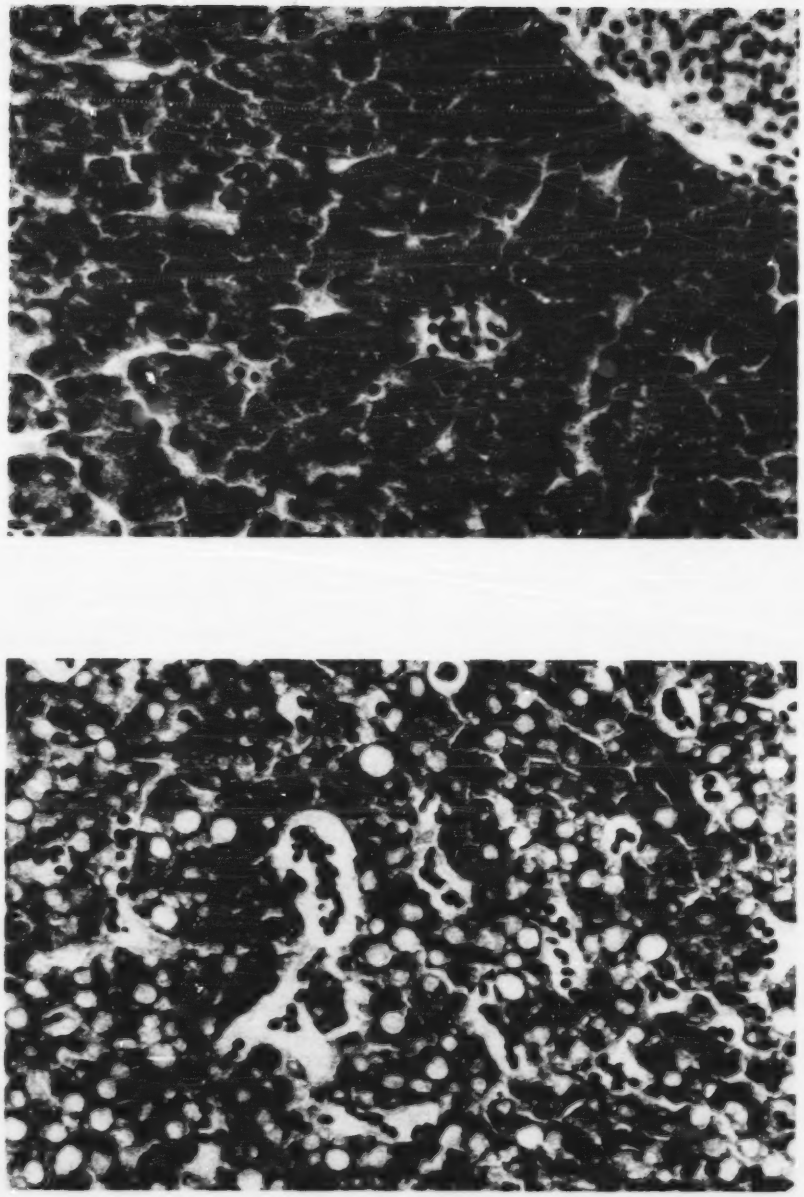

Figure 2. Upper photo: Liver of lake chub collected from control Site 1 (300 X).
Lower photo: Liver of lake chub collected from Site 2 immediately downstream of the Procter and Gamble plant, showing lipid droplets (300 X).

3.5.5 Other Findings

Free and encysted protozoa which were morphologically similar to Myxosporidia spp. were commonly found in both control and principal fish. Protozoa were found in connective tissue in the head, gills, dorsal musculature and in the brain and meninges. In most cases, there was little evidence of inflammatory reaction associated with these protozoa suggesting a well accommodated host-parasite relationship. In a few cases, small granulomata were seen, likely indicating a local reaction to dead parasites. Insufficient brain tissue was available for examination to determine the possible effects of these protozoa on the central nervous system.

3.5.6 Effects of Food Deprivation

In higher vertebrates, particularly those on a normal diet, food deprivation will result in glycogen depletion and lipid deposition (due to mobilization of lipid depots).

In this study, lake chub taken from the Wapiti River upstream of the mill were held without food for periods of time ranging from one week to 12 weeks to determine the effect of food deprivation on hepatic lipid and glycogen.

Detectable changes were not seen in either glycogen or lipid in livers taken from lake chub held at 3.5 or 9.6°C and deprived of food for periods of 1 to 12 weeks. The only detectable change was progressive atrophy of hepatocytes varying from mild at 1-2 weeks to moderate at 3-9 weeks and severe at 12 weeks.

4.0 DISCUSSION

4.1 Water Quality Parameters in Effluent and River Water

Measurement of the chemical constituents of upstream and downstream river water and mill effluent in this study was done for the purpose of correlating the chemical constituents of the water with residues in fish. As such, it does not constitute a complete study of the chemistry of either the water or the mill effluent and should be interpreted in that light. For a complete assessment of the long-term chemical characterization of mill effluent and Wapiti River water, reference should be made to existing water quality monitoring data. Nonetheless, the findings in this study are important as they relate to the findings in fish tissues. In addition, they represent a more in-depth assessment of water and effluent than is normally carried out for monitoring purposes.

A total of 47 water quality parameters were measured in upstream (control) water, mill effluent and downstream water. Forty-three parameters were detected in upstream water, 46 in mill effluent and 45 in downstream water. Both chloroform and dichlorobromoethane were detected in mill effluent but were not detected in either upstream or downstream water. In addition, dissolved organic carbon and aluminum were not detected in upstream water. All parameters except beryllium were detected in mill effluent.

The concentration of 30 water quality parameters were elevated in downstream water (in comparison to upstream water); the concentration

of 15 of these parameters showed a five-fold increase or greater. These parameters included conductivity, total dissolved solids, filterable residue, dissolved organic carbon, biological oxygen demand, chemical oxygen demand, total nitrogen, surfactants, aluminum, total mercury, potassium, sodium, chloride, sulphate and phenols.

Of these compounds, the increases in sodium, potassium and chloride were considered to be related to process chemicals while the remaining compounds were considered to be due to breakdown of tannins and lignins in the wood used as a feedstock for the mill. The increase in total mercury concentration was approximately six-fold in downstream water as compared to both upstream water and mill effluent. The elevated mercury concentrations were considered to be most likely due to physical disturbance of river sediment and not to the chemical composition of the effluent. Phenol concentrations in mill effluent were approximately 250 times greater than those in upstream water; phenol concentrations in downstream water were approximately 20 times those in upstream water.

In general, the elevation of water quality parameters in downstream water samples corresponded to the concentrations of the same parameters in mill effluent.

4.2 Organic Residues in Effluent and River Water

A total of 80 organic compounds were identified by GC/MS analysis in mill effluent and upstream and downstream water samples (Table V).

Sixty-one of these compounds were identified in mill effluent, six were identified in upstream water and 19 in downstream water.

Of the 61 organic compounds identified in mill effluent, six were acidic in nature, 16 were phenolic and 39 were neutral compounds. Thirty-one of the 61 compounds were present in concentrations of 5 µg/L or less and 16 were in the range of 6-25 µg/L. Only 14 compounds were present in the whole effluent at concentrations exceeding 25 µg/L; seven of these exceeded 100 µg/L.

Three of the 61 organic compounds detected in mill effluent were also detected upstream of the mill. These were naturally occurring fatty acids. At least three of the other acidic compounds detected in the effluent were also fatty acids, presumably of natural origin. Two of these, palmitic and stearic acids, were also detected in trace quantities in downstream water and in fish taken upstream and downstream of the mill.

Five of the 80 compounds were present in upstream water samples; all at concentrations of 1.5 µg/L or less. Four of the six compounds detected in upstream water were fatty acids, presumably of natural origin. The remaining two compounds were benzothiozole and an unidentified low molecular weight aliphatic compound.

A total of 19 organic compounds were detected in water samples taken downstream of the mill. Seventeen were detected at concentrations of 1 µg/L or less; five of these were trace concentrations. Chloroform was detected at a concentration of 2-6 µg/L and methylene chloride at a concentration of 12 µg/L.

Five of the 61 compounds identified in mill effluent were also detected in downstream water, three in trace concentrations and two at 0.5 µg/L or less. Five of the 61 compounds were also detected in tissues of fish taken downstream of the mill. These compounds were trichlorophenol (4 µg/L), tetrachlorophenol (2 µg/L), trichloroguaiacol (1.4 µg/L), α,α'-dichlorodimethylsulphone (26 µg/L) and an unidentified monochlorinated organic compound, molecular weight 122 (5.2 µg/L).

Forty-nine of the 61 compounds detected in the effluent were not found in either downstream water, or in fish tissues.

An additional three compounds were found in upstream water but not in effluent, downstream water or fish. These were natural compounds originating from plant and animal decay.

A further 12 compounds not detected in the effluent were detected in quantities of 1 µg/L or less in the downstream water except for chloroform which was detected at 2-6 µg/L and methylene chloride at 12 µg/L. Chloroform was detected by conventional chemical analysis in the effluent at a concentration of 0.71 mg/L (Table IV). It is reasonable to assume that the remaining compounds in this group (except for trichlorobiphenyl) were also of mill origin even though they were not specifically detected in the effluent. Trichlorobiphenyl was detected in downstream water at concentrations from 0.01 to 0.10 µg/L.

Only three of the 61 compounds identified in mill effluent have a half-life greater than ten days in water and can be considered persistent (Versar, 1979). These are trichlorophenol (10-15 µg/L),

tetrachlorophenol (1-15 µg/L) and tetrachloroguaiacol (1-15 µg/L). Since standards for specific isomers were not available at the time of analysis, isomeric identification of these compounds was not possible. However, subsequent analyses of effluent from this pulp and paper mill using specific isomers standards, determined that 2,4,6-trichlorophenol, 2,3,4,5-tetrachlorophenol and 2,3,4,5-tetrachloroguaiacol predominate in the effluent (J. Headley, personal communication). Trichlorophenol was detected in trace amounts in downstream water and at 4 µg/kg in intestinal fat tissue of downstream fish. Trichloroguaiacol was not detected in downstream water but was found at a concentration of 1.4 µg/kg in fat tissue of downstream fish. The remaining 58 compounds, although present at concentrations above the level of detection, are not known to be persistent in surface waters.

Chloroform was detected in the effluent at a concentration of 0.71 mg/L, about double the current drinking water standard. It was not detected in downstream water by routine analytical chemistry, but was detected at a concentration of 2-6 µg/L in downstream water by GC/MS analysis (Table V). Chloroform was not detected in fish tissues. Chloroform in water has a slow decomposition at ambient temperatures in water with a hydrolytic half-life of about 15 months and rapid volatilization loss (25 minutes to several hours depending on the degree of agitation of the water).

Dichlorobromomethane was also detected at a trace concentration in the effluent. It is produced by incomplete chlorination of alkanes

and has a maximum hydrolytic half life of 137 years with low volatilization loss from water. Specific information is not available on other fate processes such as biotransformation and sorption. Bioaccumulation may be possible (log octanol/water partition coefficient = 1.88), however, specific information has not been reported. At trace concentrations, dichlorobromomethane would not likely have an appreciable effect on downstream water quality (Versar, 1979).

4.3 Origin and Environmental Fate of Organic Compounds in Water

Persistence of organic chemicals in the aquatic environment will depend on their water solubility and vapour pressure which control the fate processes such as sorption to sediments, volatilization from the water column and biodegradation.

The origin and environmental fate of the different classes of organic chemicals listed in Table V is described below:

4.3.1 Acidic Compounds

Excepting compounds 1, 2 and 9 (Table V), the acidic compounds detected are long chain fatty acids which are probably of natural origin and result from breakdown of natural products such as vegetation and wood fibre in the water. These compounds are poorly soluble in water and physically become part of the suspended solids.

Because of their higher molecular weight and lower vapour pressure, volatilization of fatty acids will be insignificant. Hence, they tend to sorb to suspended solids and sediments leading to rapid microbial degradation. Their estimated half-lives in water would be <10 days. The remaining three compounds (1, 2 and 9) are relatively more water soluble and thus will have longer residence times than the fatty acids. However, acclimated microflora may degrade these low molecular weight acids eventually (≈30 days) (Moore and Ramamoorthy, 1984; Sittig, 1980).

Chlorobenzoic acid and 3-chlorohexanoic acid are acidic derivatives of neutral chloroaliphatic compounds and are discussed in Section 4.3.3. The origin of the unidentified aromatic acid (#2) is unknown.

4.3.2 Phenolic Compounds

The 16 phenolic compounds identified in mill effluent result from process chemicals used in the kraft process.

Alkyl-substituted phenols (1 to 4, 7 to 10, 12 and 13 under phenolic compounds in Table V) are only slightly soluble in water, whereas unsubstituted phenol has a water solubility of 6.7 g/100 ml at 16°C and a vapour pressure of 0.35 mm Hg at 25°C. Cresols (ortho-, meta- or para- methyl phenol) are less water soluble (1-17 mg/L) and have low vapour pressures (up to 1 atmosphere at 200°C). Alkyl-substituted cresols (or phenols) are all soluble in water in trace amounts only and have low vapour pressures (220-275°C to reach 1

atmosphere). Thus, volatilization will not be a significant fate process for these compounds. At the concentrations detected in this study, alkyl- substituted phenols or cresols would tend to sorb to organic-rich sediments or suspended solids and undergo microbial degradation (≈10 days) and possibly photo-oxidation in water ($t_{1/2}$ = 6000 h for p-cresol). Guaiacol (Table V, number 9) (2-methyl phenol) and other substituted phenols will undergo a similar fate due to more or less similar physico-chemical properties.

Chlorophenols are poorly soluble in water. Their volatility decreases and the melting point and boiling point increases with the number of chlorine atoms substituted into the compound. It is generally accepted that chlorophenols will undergo photolysis in water leading to substitution of Cl atoms by OH groups with subsequent polymer formation. Microbial degradation of chlorophenols has been reported in numerous studies.

4.3.3 Neutral Compounds

Excepting a few compounds, the majority of neutral compounds listed in Table IV have trace water solubility and low vapour pressure. Thus, sorption to organic-rich suspended sediments with subsequent photolysis and microbial degradation would be the predominant fate processes for their disappearance from the water column. The time taken would depend on the type and position of substituents and the physiological state of the microorganisms. It could vary from a few hours to a few days (<30 days). For example,

the photolytic half-life for benzothiophene is 34 h whereas for dibenzothiophene, it is 130 h (Haque, 1980).

Aromatic hydrocarbons such as toluene, xylene and cyclohexane (Table V, numbers 49, 50 and 23) are poorly soluble in water but have vapor pressures around 10^{-2} atmospheres. With their H (Henry's Law Constant) values around 10^{-2} atmospheres, m^3/mol., they will be moderately to readily volatile from water bodies ($t_{1/2}$ for benzene, toluene, o-xylene, naphthalene and biphenyl are 4.81, 5.18, 5.61, 7.15 and 7.52 h for a depth of one meter).

For chloroaliphatics, in spite of their relatively higher water solubility, volatilization will be the predominant fate process due to their relatively higher H values [chloroform (#46), methylene chloride (#47), carbon tetrachloride (#52), dichloroethylene (#51) and chloroaromatic hydrocarbons such as chlorobenzene (#53)].

Trichlorobiphenyl and tetrachlorobiphenyl have water solubilities of 0.25 and 0.026 µg/L respectively at 25°C. Volatilization is not the primary fate process for LCBPs (Lower Chlorinated Biphenyls), since volatility decreases with the increase in the number of Cl atoms in the rings. Tetrachlorobiphenyl (#55) degrades (<30 days) photolytically to yield trichlorobiphenyl (#54) which is resistant to further degradation. However, LCBPs (≈5 Cl) are known to be degraded by adapted microorganisms and also metabolized by small animals. PCB's strongly absorb to solid surfaces, both natural and synthetic.

α-α,dichlorodimethylsulphone (DDS) is a low molecular weight aliphatic compound which has been found in both plant and animal tissues and is readily formed by the oxidation of dimethyl sulphide. It is freely soluble in water and hence has a low bioaccumulation potential; 10^{-6} times lower than the bioconcentration potential of DDE. DDS has been shown to be not acutely toxic to fish at concentrations up to 10 µg/L (McKague, 1981) and is non-mutagenic by the Ames procedure (Voss, 1983). DDS has been identified in bleached kraft mill effluents (Lindstrom et al., 1981) and has been shown to be resistant to biological degradation. However, due to its longer residence time in water, chemical breakdown by hydrolytic and photolytic reactions will likely decay DDS to non-detectable concentrations in water. Chlorinated dimethyl sulphones are relatively resistant to biodegradation but may undergo volatilization.

4.4 Fish Studies

4.4.1 Assessment of Toxic Effects in Fish

Although a direct estimate of fish populations above and below the discharge site was not carried out, all other data indicated that effluent from the mill was not acutely toxic to fish resident in the area. Other factors which were considered in assessing the toxic potential of the effluent under natural conditions included species diversity above and below the mill, apparently satisfactory reproductive activity as determined by the presence of young-of-the-year, relative ease of capture of fish above and below

the mill and the absence of overt evidence of mortalities. These observations are consistent with the results of 96 hour LC_{50} tests of the mill effluent conducted in this laboratory over several years using juvenile rainbow trout (Salmo gairdneri). For example, of seven tests conducted since 1978, only two resulted in 100% mortality and this was due to low oxygen concentration. The low oxygen content of the effluent sample could originate from several causes including seasonal changes in water quality or chemical or bacterial contamination during sampling or storage.

No long term (subacute or chronic) laboratory studies of this effluent have been carried out. However, a detailed examination of fish with little migratory activity and which are resident downstream of the effluent discharge can provide some indication of the effects of "chronic" exposure. In this study, no grossly visible evidence of abnormalities was found. As well, the presence of the young-of-the-year in the vicinity of the effluent discharge suggested that reproduction was not impaired.

4.4.2 Analysis of Fish Tissues for Organic Residues

A total of seven compounds was detected in homogenates of whole fish resident upstream or downstream of the mill effluent diffuser. Two of these compounds, palmitic and stearic acids, were detected in both upstream and downstream fish and are fatty acids of natural origin. Five compounds were detected in downstream fish but not in upstream fish. Each of these five was also detected in mill

effluent. Two compounds, trichlorobiphenyl and tetrachlorobiphenyl were detected in trace amounts in upstream fish but not downstream fish. Tetrachlorobiphenyls (PCB's generally) are ubiquitous in nature and derived from a variety of industrial uses.

The collective, and particularly, the long term significance of the organic chemicals detected in the effluent is not known. The fact that two-thirds of the compounds detected were not found either in downstream water or in fish suggests that they are not persistent and would have little effect on fish populations or on downstream water quality.

4.4.3 Analysis of Fish Tissues for Pesticides and PCB's

Homogenates of whole fish resident in the Wapiti River upstream of the mill effluent diffuser yielded trace concentrations of trichlorobiphenyl and tetrachlorobiphenyl by gas chromatography/mass spectrometry. Trichlorobiphenyl had also been detected in downstream water samples but was not detected in upstream water or fish taken downstream of the mill. Both trichlorobiphenyl and tetrachlorobiphenyl are classified as lower chlorinated biphenyls. Trichlorobiphenyl is a breakdown product of tetrachlorobiphenyl which is resistant to further degradation and therefore is persistent in aquatic environments.

The presence of three species of Aroclor, a commercial form of polychlorinated biphenyls, was confirmed in intestinal fat samples of downstream fish at ~ 5 µg/kg. Polychlorinated biphenyls generally derive from a variety of industrial uses. They are widespread and

persistent background contaminants and their detection in this study is not directly related to mill effluent. However, a PCB spill did occur at the plant in 1981 and may have contributed to the presence of these compounds.

DDT and DDE were also detected in fat tissue of fish resident upstream and downstream of the mill at concentrations close to the detection limit of 5 µg/kg (ppb). DDT and its metabolite DDE are persistent organochlorine insecticides which have resulted from widespread generalized used of DDT as an insecticide. Forty other common pesticide species of several different chemical classifications were not detected in fish tissue either above or below the mill site. The presence or absence of these compounds is also unrelated to mill effluent.

4.4.4 Pathological Examination of Fish Tissues

Except for relative differences in hepatic glycogen and lipid content, no differences were seen between upstream (control) and downstream (principal) fish by histopathological examination at the light microscopic level. Hepatic glycogen and lipid may vary considerably with nutritional status and different stages of reproductive and life cycles (Cowey and Roberts, 1978). Most of the differences observed in this study were subtle; thus these changes must be interpreted in light of observations in control fish. In a few cases, the lipidosis was pronounced.

4.4.5 Glycogen Metabolism in Fish

Glycogen is the storage form of glucose. Glucose, in fish as in mammals, is the basic unit of chemical energy utilized in aerobic metabolism and the liver is the principal site of glucose metabolism.

Glycogen metabolism in fish is less well defined than in mammals (Cowey and Roberts, 1978). Most of the chemical pathways involved in glycogen metabolism in fish are similar, but not always identical, to those in mammals. In some species, for example, epinephrine and glucagon do not control glycolysis. The effect of starvation on glycogen depletion is not as clear as some species regularly undergo seasonal dormancy and inanition. The effects of migratory patterns and reproductive cycles on glycogen status are also not well understood.

In this study, observable glycogen content in lake chub downstream of the mill was about half that of upstream fish. More than half of the upstream fish had clearly visible glycogen in the hepatocytes. However, only three of 22 downstream fish had clearly observable hepatic glycogen and most had no visible glycogen at all. This observation suggests that glycogen metabolism may have been modified in some way by the mill effluent.

This difference in observable hepatic glycogen was much less pronounced in the longnose sucker. Glycogen content of downstream fish was approximately two-thirds that of upstream fish. The differences between these two species may represent slight variations in glycogen metabolism, different feeding habits, or, alternately, a greater resistance to the effects of the effluent.

Changes in observable hepatic glycogen in lake chub were not seen in the food deprivation experiment carried out as part of this study. No differences were seen as a result of starvation for periods up to 12 weeks at 3 or 10°C. The only change observed was a progressive atrophy of hepatocytes. This may have been due to the fact that the glycogen content of the hepatocytes was not high at the beginning of the starvation period. The behaviour of the fish during the starvation period did not seem to be affected by prolonged food deprivation suggesting a much higher tolerance than would be the case in higher vertebrates.

4.4.6 Lipid Metabolism in Fish

Normal intracellular fat exists in adipocytes in body fat depots and in organs actively involved in lipid metabolism of which the most important is the liver (Cheville, 1983; Jubb and Kennedy, Vol. 2, 1970). Intracellular fat is comprised of one or more locules of triglycerides which are synthesized in the cell from free fatty acids which are the transport forms of lipid. Normal lipogenesis and lipolysis are influenced by a variety of genetic, dietary and hormonal factors. Continuous deposition and mobilization are normal events even in adipocytes. Lipid is used for immediate energy production, as a storage form of energy and as a building block for products such as phospholipids, sphingolipids and lipoproteins.

There is no evidence that normal lipid metabolism is markedly different in most species of fish from that of higher vertebrates.

However, in some species of wild fish, extreme seasonal lipid infiltration occurs and is not considered abnormal (Cowey and Roberts, 1978).

Abnormal lipid metabolism occurs, for the most part, in the liver, although in severe circumstances, other tissues such as kidney and myocardium may be involved. Abnormal lipid metabolism usually means excessive accumulation of lipid. A complete absence of lipid would be abnormal in the sense that most hepatocytes have some but this former condition would be rare except in chronic starvation states.

Abnormal accumulation of lipid may occur by two general mechanisms; 1) a prolonged over-supply of fatty acids, and/or 2) under-utilization of triglycerides (Cheville, 1983). An oversupply of fatty acids implies an excessively high level of blood lipid for an extended period of time. This condition is generally associated with prolonged excessive dietary lipid or a chronic metabolic disease such as diabetes mellitus, pregnancy toxaemia or ketosis (Froesch, 1979). An under-utilization of triglycerides implies some impairment in lipid metabolic pathways which may vary from very mild to severe and from transient to permanent. Impairment of lipid metabolism is usually of toxic origin and may be due to prolonged hypoxia or direct interference with enzyme systems by some toxic agent.

4.4.7 Hepatic Lipidosis as an Indication of Toxic Injury to Fish

Because the liver is the principal and most active metabolic organ in the body, lipid accumulation is, in the absence of known dietary

excess or metabolic disease, an early non-specific indicator of toxic injury. There is, however, no clear distinction between a degree of injury which may be transient, physiological and reversible and one that may be pathological. The difference is one of extent, degree and distribution. Some interpretation can often be drawn from the distribution of the lipid in the liver, the size and number of lipid locules and the appearance of other tissues. It is also necessary to keep in mind that dose and duration are essential factors of any toxic effect.

Available evidence suggests that the factors governing normal and abnormal lipid metabolism in fish are not markedly different from those of higher vertebrates (Cowey and Roberts, 1978; Hochachka, 1969). Cowey and Roberts have reported that, at certain times of the year, extreme lipid infiltration is common in some species of wild fish and is not considered abnormal. The extreme lipid changes associated with poor quality or rancid diet have also been reported and are referred to as lipoid liver disease. Starvation, however, has not been reported to produce hepatic lipidosis in fish.

Walsh and Ribelin (1975) reported that non-specific changes in liver morphology were frequently associated with pesticide exposure. Fatty infiltrates (among other changes) were shown in experimental exposures using lake trout and coho salmon. In these studies, hepatic lipidosis was a common finding after exposure to endrin, Na arsenite, DDT, Carbaryl, Malathion, Endosulfan, Dieldrin and 2,4-D. Lipid was consistently present in the liver of exposed fish and not in those of

controls. Couch (1975), working with wild spot (Leiostomus xanthursus), a marine species, reported that hepatic lipidosis was the most commonly encountered non-specific liver lesion following pesticide exposure. Lipid was not present in livers of wild untreated spot. Couch also reported impairment of sensory systems (lateral line) associated with a variety of environmental contaminants including pulp and paper effluents.

In the present study, visible hepatic lipid was not found in any of the 16 lake chub or 14 longnose sucker taken upstream of the mill (control fish). However, ten of 22 downstream lake chub did show observable lipid including three animals in which liver lipid was judged to be moderate to severe. In contrast, only three of 14 longnose sucker taken downstream of the mill showed evidence of hepatic lipid and these were of a lesser degree than the lake chub.

The presence of hepatic lipid in fish collected downstream of the mill as opposed to upstream controls does suggest that the mill effluent may have had some effect on these animals. However, the mild degree of lipidosis and the absence of other negative factors suggest that this effect was probably minimal. Differences in both glycogen and lipid levels between the two species studied also suggests that the sucker are much less sensitive to these changes than the lake chub.

5.0 CONCLUSIONS

5.1 Effluent from the mill was often not acutely toxic to fish under natural or laboratory conditions.

Twelve species of fish were found immediately below the waste discharge point compared with ten species in the upstream control area. The age distribution of the species caught above and below the discharge point was similar. Based on the presence of young-of-the-year, at least seven species of fish appeared to reproduce in the vicinity of the mill. Acute toxicity tests of whole mill effluent conducted over a period of several years were often negative.

5.2 Effluent from the mill contains alkanes, fatty acids, phenolic compounds, dissolved solids, chemical oxygen demand and biochemical oxygen demand all commonly associated with pulp mill effluent. Discharge of these constituents lead to a measurable increase in their concentrations in the Wapiti River immediately below the mill.

5.3 Fish captured 0.01 - 2.5 km below the mill contained alkanes, fatty acids, chlorophenols and guaiacols, and α,α'-dichloro-dimethylsulphone in their tissues at concentrations ranging from trace (detected by not quantifiable) to 5 µg/kg (ppb, wet

weight, whole fish), except for α,α'-dichlorodimethyl-sulphone which was detected at a concentration of 26 µg/kg. In most cases these compounds were also detected in mill effluent; in a few cases they were also detected in upstream water samples and in fish taken upstream of the mill.

5.4 Fish captured upstream and downstream of the mill contained concentrations of polychlorinated biphenyls in intestinal fat (but not muscle tissue) at concentrations ranging from trace (detected but not quantifiable) to 5 µg/kg (ppb). Intestinal fat samples of fish captured downstream of the mill also contained similar concentrations of DDT and DDE. These compounds were not detected in mill effluent and the concentrations detected in fish tissues were well below those currently accepted as safe for human consumption.

Forty other common organic environmental contaminants, primarily pesticides, were analyzed for but not detected in intestinal fat or muscle tissue from fish taken downstream of the mill.

5.5 Histopathological examination of tissues of small fish resident downstream of the discharge point revealed a slight increase in observable lipid in the livers. This finding suggests a mild unspecified impairment of lipid metabolism and possibly of protein synthesis. The effect of this change on fish production in the river is not known.

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APPENDICES

APPENDIX I

Analytical Methods for Effluent and Water Samples

Extraction of Base/Neutral (B/N) Fraction

One litre of the sample was transferred into a 2 L separatory funnel and rinsed with 60 ml of pure (glass-distilled) methylene chloride ($MeCl_2$). The aqueous layer was adjusted to pH 13 with NaOH and demulsified, if needed, with saturated NaCl solution. The $MeCl_2$ layer was collected in an Erlenmeyer flask. The extraction was repeated with another 60 ml of $MeCl_2$ and the extracts were combined. The $MeCl_2$ extract was dried with organic-free Na_2SO_4 and the Na_2SO_4 was then washed with 25 ml $MeCl_2$. The combined $MeCl_2$ extract was concentrated to 1 - 2 ml in a rotary evaporator and the concentrate was transferred to a volumetric flask, followed by two rinses of the evaporating flask with $MeCl_2$. The extract was spiked with 1 µg of d_{10} phenanthrene and concentrated to 50 µl with dry N_2 gas. Then the extract was transferred to a 100 µl graduated vial with a suitable syringe followed by two rinses of the flask with 25 µl of hexane. The sample was concentrated, if necessary, with dry N_2 gas to a final volume of 100 µl. This was the B/N fraction.

Extraction of Acidic (A) Fraction

The aqueous phase in the separatory funnel was acidified with concentrated H_2SO_4 to pH ≤ 2 and then extracted serially three times with 60 ml (per extraction) of distilled-in-glass $MeCl_2$. The extracts were combined in an Erlenmeyer flask and dried by passing through a column of acidified anhydrous Na_2SO_4 (pH 4). The flask was rinsed with 20 - 40 ml of $MeCl_2$, collected through the Na_2SO_4 column and combined with the remaining $MeCl_2$ extracts. The combined extract was then concentrated to 1 - 2 ml in a rotary evaporator and transferred to a volumetric flask along with two rinses of 2 ml each of $MeCl_2$. The extract was spiked with 1 µg d_{10}-phenanthrene and concentrated, as described for the B/N fraction, to 100 µl volume in a graduated vial. This was the acidic (A) fraction.

Analysis of Extracted Water and Effluent Samples

The base/neutral and acidic extracts were analyzed by GC/MS, identifying and quantitating all compounds >1.0 µg/L. Halogenated compounds <1 µg/L in concentration were also identified. Compounds were identified from the interpretation of their mass spectral fragmentation pattern and also by comparison with reference spectra (Heller and Milne 1978; Mass Spectrometry Data Centre 1975; Stenhagen et al. 1974). Quantitation was performed using known response factors and in cases where authentic standards were not available, a value of unity was assigned to the response factor.

APPENDIX II

Analysis of Fish Tissues for Organic Residues

Samples of fish were prepared for organic analysis by extraction to produce acidic, basic and neutral fractions (Method I) and subsequently base-neutral and acidic fractions (Method II). Most fish weighed approximately 0.6 g with a few weighing up to 10 g. In most cases, fish were pooled by species and location to arrive at a sample size of ~9.0 g. The whole fish was cut into small pieces and blended with acetone in a mechanical blender. The homogenate was then concentrated to dryness.

Method I (Figure 3) separated the extract into three fractions; acidic, basic and neutral fractions. Initial analytical experience showed that compounds identified under "basic" were mostly carry-overs from the neutral fraction, except for identifying compounds such as ethyl benzene, 1,4-dimethyl benzene, dimethyl trisulfide and di-2,ethylhexyl phthalate. Due to a very low response from the analyte compared to a high background noise, most peaks could not be identified without major clean-up of the extract. This problem, common to many laboratories, led to the development of Method II which pooled the basic and neutral fractions into one fraction called base/ neutral, without sacrificing sample integrity.

Method II was a modification of the method of Longbottom and Lichtenberg (1982) where a liquid-liquid extraction technique was used instead of column chromatography (Figure 3). This method saved considerable instrument (GC/MS) time in the analysis without compromising compound identification.

EXTRACTION OF FISH TISSUE (Method I)

WHOLE FISH, BLEND WITH ACETONE
CONCENTRATE TO DRYNESS
(i) ACIDIFY WITH CON. H_2SO_4 (pH, <2)
(ii) ADD NaCl SOLUTION
(iii) EXTRACT WITH CH_2Cl_2 (150+50+50 ml.)
CH_2Cl_2 LAYER OAN SOLUTION
AQUEOUS LAYER pH, <2
EXTRACT WITH 0.2 KOH (200+200ml.)
(i) ADD 2N NaOH (pH, >12.5)
(ii) EXTRACT WITH CH_2Cl_2 (150+50+50 ml.)
CH_2Cl_2 LAYER ON SOLUTION
AQUEOUS LAYER pH = 12.5
CH_2Cl_2 LAYER OB SOLUTION
AQUEOUS LAYER DISCARDED
(i) WASH WITH 0.1 N HCl
(ii) DRY OVER Na_2SO_4
(i) ACIDIFY (pH, <2)
(ii) EXTRACT WITH CH_2Cl_2 (50+50+50 ml.)
(i) DRY OVER Na_2SO_4
NEUTRAL FRACTION
CH_2Cl_2 LAYER OA SOLUTION
AQUEOUS LAYER DISCARDED
BASIC FRACTION
(i) CLEAN UP WITH FLORISIL COLUMN
(ii) ELUTE WITH EtOAc/pE (40/60 Mixture)
(i) DRY OVER Na_2SO_4
(ii) METHYLATE WITH CH_2N_2
CLEANED NEUTRAL FRACTION
METHYL ESTERS OF ACIDIC FRACTION

Extraction and Clean-up of Fish Tissues

The whole frozen fish was cut into small pieces and allowed to thaw to room temperature. Five grams of the minced whole sample was weighed and homogenized in a sonicator with 75 ml of acetone/hexane (2:1) mixture. After the homogenate settled (~5 minutes), the organic layer was decanted into a 2 L separatory funnel. The residue was homogenized again in 30 ml of hexane/ether (2:1) mixture and 4 ml of HCl (0.1N) solution for 30-45 minutes. The organic layer was decanted and pooled with the earlier one. The residue was homogenized again twice with 30 ml of hexane/ether (1:1) mixture and finally with 50 ml of the same mixture. The organic layer was decanted and added to the earlier ones in the separatory funnel. The volume of the extract at this stage was ~200 ml. The residue is then rejected.

One litre of organic-free deionized distilled water was added to the organic extract in the separatory funnel and NaOH (0.2M) solution was added slowly to raise the pH to >13. Any emulsion formed was removed by adding 75 - 150 ml of saturated NaCl solution and then shaking with 30 ml of hexane/diethyl ether (1:1) mixture. This was followed by two more extractions with 30 ml each of the same solvent mixture. All the organic extracts were combined in a 250 ml flask and prescreened by GC. The extract was dried with organic-free Na_2SO_4 with subsequent washing of Na_2SO_4 with diethyl ether (10 - 15 ml). Then the extract was concentrated on a rotary evaporator to ~0.5 ml (not to dryness) and transferred quantitatively into a 2 ml volumetric flask. The 250 ml flask was rinsed twice with 100 µl

each of hexane/ether mixture and rinsings were added to the 2 ml flask. The content was then spiked with 5 µg of d_{10}-phenanthrene and concentrated, if necessary, to a final volume of 2 ml with dry N_2 gas or adjusted with pure hexane. This was the B/N fraction of the fish extract.

The aqueous phase was acidified with concentrated H_2SO_4 (pH $\leq$2) and extracted serially three times with 30 ml each of hexane/diethyl ether (1:1) mixture. The combined extract was dried through acidified Na_2SO_4 (pH 4) and rinsed with 10 - 15 ml of the same solvent mixture. It was then concentrated to ~0.5 ml on a rotary evaporator, transferred to a 2 ml volumetric flask and processed similarly to the B/N fraction to arrive at the acidic fraction.

The concentrated Acidic and B/N fractions (~0.5 ml) were transferred to separate columns of 10 g each of Florisil (3% water deactivated). These were sequentially eluted with (i) 150 ml of hexane (Fraction I), (ii) 150 ml of hexane-dichloromethane (1:1) mixture (Fraction II), (iii) 200 ml of dichloromethane (Fraction III) and (iv) 200 ml of methanol (Fraction IV). Fractions I and II were cleaner fractions of the extract and the 'fats' were in Fraction IV.

Analysis of Fish Tissues

Extracts were analyzed by Gas Chromatography/Mass Spectrometry using the "Selected Ion Monitoring" (SIM) methodology (Kenyon, 1981). All solvents were pesticide grade, glass-distilled and were redistilled prior to use. Quality assurance precautions included

instrument calibration with conventional standards, recovery studies from spiked samples and process and field blank determinations. An internal standard was added to every extract.

The mass spectrometer was calibrated with PFTBA (perfluorotributylamine) and quantitation of the organics was performed using an internal standard, d_{10}-phenanthrene. Most priority pollutants (57%) were recovered in the range 60-99% with a precision of less than 20% standard deviation (Eichelberger et al., 1983; U.S. EPA, 1982).

Analysis was performed using a Hewlett-Packard (HP) model #5987 GC/MS fitted with a HP model #5840 gas chromatograph, equipped with a 0.25 mm ID x 25 m OV-1 fused silica capillary column of 33 μ film thickness. A helium linear velocity of 25 cm/second was employed with a splitless mode injector. The column was directly coupled to the mass spectrometer and the oven temperature was programmed at 50°C for 1 minute followed by an 8°C/minute temperature increase to 280°C. The injector and ion-source temperatures were 300°C and 200°C respectively. The electron energy was 70 eV.

APPENDIX III

Analysis of Fish Tissues for Pesticides and Polychlorinated Biphenyls

Extraction Procedures for Muscle and Fat Tissues

Twenty-five grams of the muscle tissue free from bone, skin and scale was ground with a Polytron homogenizer in a 250 ml beaker in the presence of 100 ml of distilled-in-glass acetonitrile. It was then filtered in vacuum and the filtrate transferred into a 1 L separatory funnel with 150 ml of petroleum ether (PE). Six hundred ml of PE (washed with distilled water) and 5 g of NaCl were added to the filtrate and shaken well. The PE layer was separated, dried through anhydrous Na_2SO_4 and evaporated to near-dryness. The moist residue was reconstituted with a minimum amount of PE and processed by column chromatography. A chromatography column with 50 g of Florisil containing 5% water was prepared and the concentrate was applied onto the column. Two hundred ml of PE was poured onto the column and the "Petroleum Ether Fraction" (Fraction I) was eluted. Then 275 ml of PE containing 25% $MeCl_2$ was poured in and Fraction II was eluted.

Each fraction was evaporated to near-dryness and reconstituted with a small volume of hexane (5 - 10 ml). A small portion (e.g., 5 µl) of this solution was then injected to a gas chromatograph with an electron capture detector.

One gram of fat was weighed and ground in a mortar with 25 g of Florisil containing 5% water and some anhydrous sodium sulfate. A chromatography column was prepared with 25 g of Florisil containing 5% water, then the above sample with Florisil was added. The PE Fraction

I was obtained by eluting with 250 ml of PE and the Fraction II was obtained by eluting with 275 ml of PE containing 25% methylene chloride. Both fractions were then evaporated to near dryness, reconstituted with hexane and an appropriate volume injected into the gas chromatograph as previously described for the muscle tissues.

Perchlorination of PCB for Reduced Detection Limit

Extracted fractions and a standard decachlor biphenyl were transferred to separate Teflon-capped vials with ~0.2 ml of PE and immersed in a warm water bath. The PE was evaporated slowly with an airstream (just to dryness) and 0.2 ml of antimony pentachloride ($SbCl_5$) was added. All vials and a blank vial were capped and placed in a GC oven overnight at 180°C.

The vials were then cooled to room temperature and immersed in an ice-bath for several minutes before adding ~2 ml of 6N HCl. After swirling the bath, ~2 ml of $MeCl_2$ were added and the vials were shaken for several minutes. When the layers separated, the top $MeCl_2$ layer was drawn off with a Pasteur pipet into a test tube containing 3-4 ml of saturated $NaHCO_3$ solution. The acid layer was extracted two more times with $MeCl_2$ and extracts combined and shaken with $NaHCO_3$ solution in the test tube. The $MeCl_2$ layer was extracted once more with 2 ml of $MeCl_2$ and transferred to the Florisil column. The column was then eluted with 20 ml of 25% $MeCl_2$ in PE. Solvent was then evaporated to reach a desired volume for analysis by a GC fitted with an electron capture detector.

Analysis of Extracted Tissues for Pesticides and PCB's

Approximately 5 µl of Fractions I and II of both muscle and fat tissue were injected into a gas chromatograph with an electron capture detector. The gas chromatogram obtained was compared to a standard pesticide gas chromatogram run along with the sample. The concentrations of pesticide in the sample were calculated by comparing the peak height of each pesticide to the peak height of the respective standard. Analysis of the non-perchlorinated samples also provided a semi-quantitative screen for high concentrations of PCB's.

Fraction I would be expected to contain very non-polar compounds such as HCB, DDE, DDT and PCB. Fraction II would be expected to contain slightly polar compounds such as α, ß, γ-BHC, HE, Dieldrin, Endrin and DDD. Each fraction was evaporated to near-dryness and reconstituted with a small volume of hexane (5 - 10 ml). A small portion (e.g., 5 µl) of this solution was injected to a gas chromatograph with an electron capture detector.

Following perchlorination, Fractions I and II of both muscle and fat tissues were reinjected into the GC. For this purpose the GC was fitted with a 12 inch glass column packed with QF-1/OV-1 with ratio of 2:3, with oven temperature set at 220°C.

This method of perchlorination saturates the PCB with respect to chlorine producing decachlorobiphenyl. The electron-capture detector has a high sensitivity for decachlorobiphenyl and thus is able to achieve a lower level of detection. Quantitation of samples is done by matching sample peak and perchlorination with the standard

instrument calibration with conventional standards, recovery studies from spiked samples and process and field blank determinations. An internal standard was added to every extract.

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decachlorobiphenyl. Precision and accuracy for this method is given below:

Precision and Accuracy:

1. Detection limit (based on 25 g samples)
   - organochlorines, 0.001 ppm
   - PCB's 0.2 ppm (without perchlorination)
   - 0.002 ppm (with perchlorination)
2. Recoveries from fortified fish muscle
   - organochlorines and PCB's, >80%
3. Accuracy:

Concentration of PCB's/organochlorine (mg/kg)	Limit of accuracy
1.0	± 10%
0.1	± 20%
0.01	± 50%
0.001	±100%

4. Reproducibility (Precision);
   - ± 10%

GLOSSARY OF TERMS USED

A = Acidic fraction

BKME = Bleached kraft mill effluent

γ-BHC = γ-isomer of hexachlorocyclohexane, commercially known as lindane

B/N = Base - neutral fraction

°C = Degree centrigrade

DDD = 4,4'-dichlorodiphenyl dichloromethane, $C_{14}H_{10}Cl_4$

DDE = 4,4'-dichlorodiphenyl dichloroethylene, $C_{14}H_8Cl_4$

DDT = 4,4'-dichlorodiphenyl trichloroethane, $C_{14}H_8Cl_5$

Dieldrin= Member of cyclodiene group of insecticides

Endrin = Common name of one member of cyclodiene group of pesticides

eV = Electron volt

HCB = Hexachlorobenzene

HCl = Hydrochloric acid

ID = Internal diameter

km = Kilometer

m^3= cubic meter (m^3 in equations)

$MeCl_2$ Methylene chloride

mg/L = Milligram per litre

N_2 = Nitrogen gas

NaCl = Sodium chloride

NaOH = Sodium hydroxide

Na_2SO_4 = Sodium sulphate

PCB's = Polychlorinated biphenyls

PE = Petroleum ether